

CAROTENOID PIGMENTS IN THE CELLULAR SLIME MOLD, *DICTYOSTELIUM DISCOIDEUM*^{1, 2}

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Five distinct stages of development may be recognized in the life-cycle of the cellular slime mold, *Dictyostelium discoideum* (Bonner, 1944; Raper, 1937, 1939, 1940). The first four stages are relatively colorless, but the fifth, or fruiting body, stage is marked by a change in the color of the sori from pale buff to bright lemon yellow. Since a change in color in an organism may reflect changes in metabolic events, the nature of the pigments was investigated to determine: (1) whether this color transition represents *de novo* synthesis by the spore cells or the mere accumulation of substrate pigments from the medium, as suggested by Whittingham and Raper (1956); (2) the nature of the pigment; (3) the effect of diphenylamine, which specifically inhibits carotenogenesis (Goodwin, 1952, 1954; Haxo, 1955; Kharasch, 1936; Turian, 1950; Zalokar, 1957).

METHODS AND MATERIALS

Culture procedures and harvesting of tissue

All cultures of *D. discoideum* and the bacterial associate, *Escherichia coli*, were maintained on an agar medium (Bonner, 1947) in Petri plates. Cultures were incubated in darkness at 22° C. in an environment ranging from 55 to 90% relative humidity. Following various intervals of incubation, cultures of *D. discoideum* were harvested for dry weight determination and pigment assay. Cultures were scraped into tared flasks and weighed wet. An aliquot of known wet weight was removed for dry weight determination and the remaining wet sample was extracted for pigment. The aliquot for dry weight determination was freed of *E. coli* in a 0.55 M/0.95 M sucrose gradient, washed, dried, and weighed. This procedure permitted calculation of the dry weight of the sample used for pigment extraction.

In order to extract the pigment, cultures of the desired stages were scraped into 95% ethanol, allowed to stand several hours, and filtered. The residue, from greyish-white to pale yellow in color, was discarded, and the yellow filtrate (ethanol extract) was stored in darkness at 10° C. Extracts obtained by this procedure were either assayed for pigment as described below or purified further.

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Assay of pigment

The optical density (O. D.) at 390 $m\mu$ of ethanol extracts was proportional to the O. D. at 390 $m\mu$ of the more purified preparations described later, and was suitable for gross quantitative assay of pigment.

Purification of pigment for analysis

When large quantities of pigment were needed for chemical analyses, only cultures of mature sorocarps were harvested, and were extracted as described previously. Cultures containing only *E. coli* and nutrient agar contained no pigment and were not further studied. The ethanol extract was saponified with 10% KOH (w/v) at 65° C. for 2 hours. Following hydrolysis, the pigment solution was transferred to a separatory funnel with an equal volume of diethyl ether and sufficient water to effect separation of the two phases. Acetic acid was added to transfer all pigment into the ether phase. The epiphase was then washed with water, dried over anhydrous Na_2SO_4 , transferred to an Erlenmeyer flask, and taken to dryness under reduced pressure (Residue I). Small portions of hexane were added to Residue I and then decanted from the flask until further additions of solvent remained colorless. The hexane extracts were combined, reduced in volume under vacuum, and poured onto a powdered sucrose column. The column was first developed with hexane, and then washed successively with diethyl ether and methanol. The major yellow fraction was eluted with ether, and is designated Fraction I. The hexane eluate, which was also yellow, was rechromatographed on MgO: Celite (1:1). Several pigments were subsequently eluted from this column with hexane and ether, but the very small quantities present precluded further analysis. Residue I was subsequently extracted with ether, and the ether-soluble pigments were also chromatographed on powdered sucrose. Developing the column with ether eluted a pigment designated Fraction II-a. The column was then washed with methanol, eluting Fraction II-b. Following removal of the hexane and ether-soluble pigments from Residue I, the remaining pale yellow residue was taken up in methanol (Fraction III).

Chemical analyses of fractions

The presence of an acidic function was tested by noting differences in the distribution behavior of the salt and the free acid between two solvents (Fox, 1953; Zalokar, 1957). The absorption spectra of the acidic and basic forms of the pigment were also compared (Zalokar, 1957).

Several qualitative color tests were used to detect polyene structure. Fractions I through III were taken to dryness, and a drop of concentrated H_2SO_4 was added to the residues. In addition, concentrated H_2SO_4 was layered under ether solutions of Fractions I and II-a (Karrer and Jucker, 1950). A few crystals of dithionite, a reducing agent, were added to Fractions I and II-a in ether and to Fractions II-b and III in methanol. Color changes were noted. Antimony trichloride was added to chloroform solutions of Fractions I and II-a (Carr and Price, 1926; Karrer and Jucker, 1950). This reaction could not be carried out on Fractions II-b and III because they were insoluble in chloroform.

Spectrophotometric analyses

All spectral data were obtained with either a Beckman DK-2 or a Bausch and Lomb Spectronic 505 recording spectrophotometer.

The effects of diphenylamine

Culture media were prepared by adding to the agar a stock solution of 10^{-2} M diphenylamine (DPA) in 95% ethanol to give concentrations of DPA from 5×10^{-6} M to 5×10^{-5} M. Controls were prepared by adding the appropriate volume of ethanol to the culture media. Plates were then inoculated, incubated for various intervals, and examined for relative number of sorocarps and intensity of coloration.

RESULTS

Studies relating pigment concentration to developmental stage

The course of pigmentation is shown in Figure 1. The concentration of pigment was low initially and did not change appreciably for 60 hours. It then increased slowly until fruiting began at about 72 hours. At 84 to 96 hours, fruiting was morphologically complete and the pigment concentration continued to increase. Major pigment accumulation did not occur until after fruiting was complete.

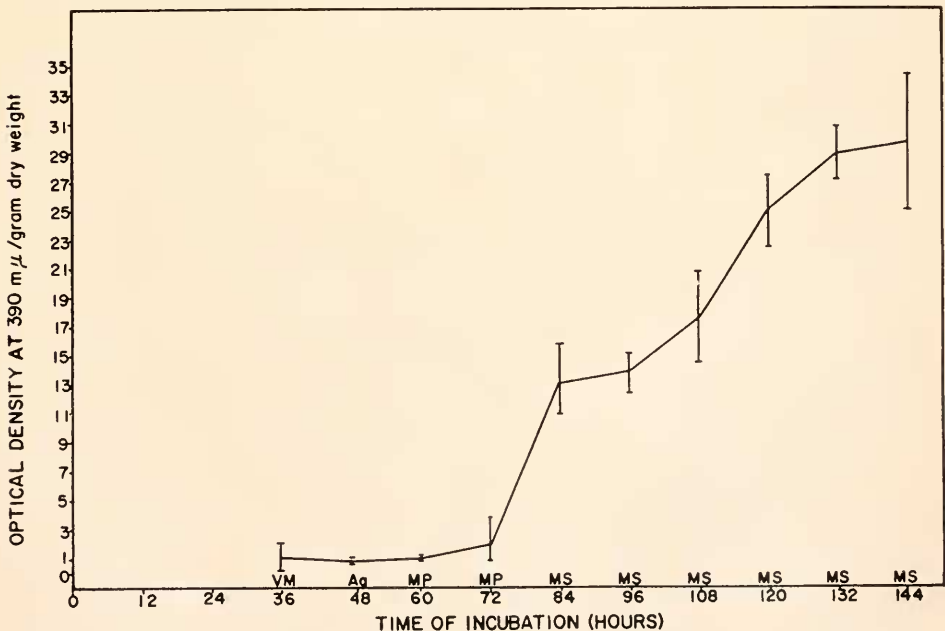


FIGURE 1. Increase in pigment concentration during development. VM = Vegetative myxamoebae. Ag = Aggregates. MP = Migrating pseudoplasmodia. MS = Mature sorocarps.

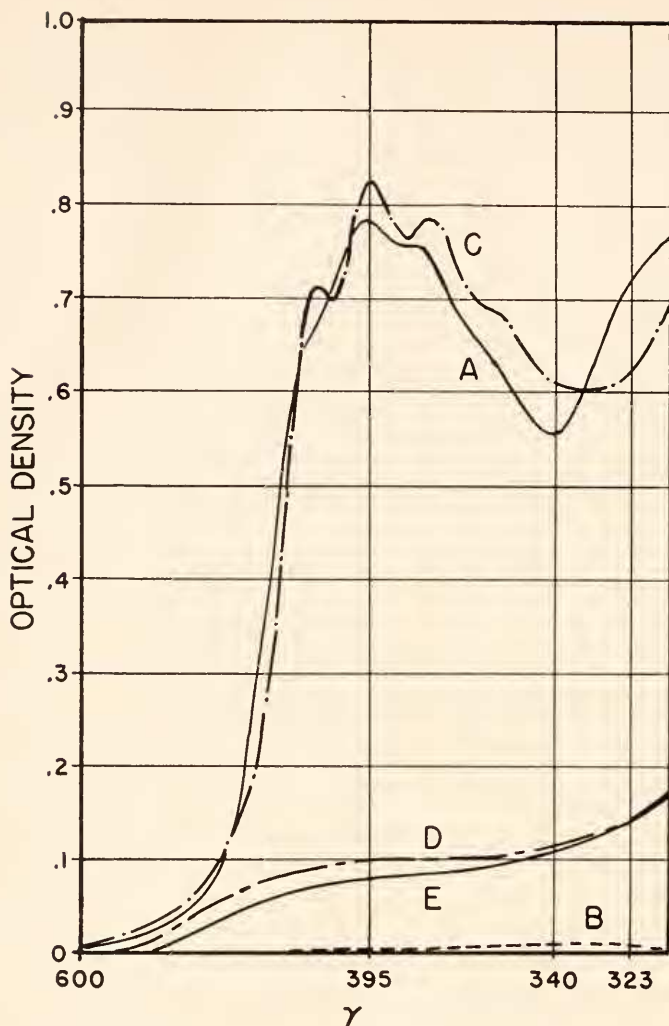


FIGURE 2. Absorption spectra of *D. discoideum* and *E. coli* extracts. A = *D. discoideum* ethanol extract. B = *E. coli* ethanol extract. C = *D. discoideum* ether phase. D = *E. coli* ether phase. E = Control hydrolysate.

Comparison of spectra of *D. discoideum*, *E. coli*, and nutrient agar ethanol extracts

The ethanol extracts of *D. discoideum*, *E. coli*, and nutrient agar were examined spectrophotometrically. The *D. discoideum* extract showed a peak at 398 $m\mu$ with shoulders at 414 $m\mu$ and 381 $m\mu$. The spectra of the *E. coli* and agar extracts showed no absorption of the visible region (cf. Curves A and B, Figure 2). Further, the ether phases obtained after hydrolysis of ethanol extracts of *D. discoideum*, *E. coli*, and agar were compared. As shown in Figure 2, the strong absorption band seen in the ethanol extracts of *D. discoideum* was resolved to yield absorption

maxima at 417 $m\mu$, 397 $m\mu$, and 377 $m\mu$ (Curve C). On the other hand, the spectra of the colorless *E. coli* and agar extracts were essentially the same as that of the control.

Spectral characteristics of pigment fractions

The absorption maxima of the pigment fractions are summarized in Table I.

Chemical tests

Test for an acidic function

Partitioning an ether solution of Fraction I against 2 N NaOH moved the pigment to the interface where it was visible as a yellow layer. Addition of methanol distributed the pigment between the two phases. Acidification restored the pigment to the ether phase.

TABLE I
Comparison of literature and experimental maxima at λ_1

Compound	Solvent	Maxima at λ_1				
zeta-carotene ^a	Hexane	425	400	378	360	295
zeta-carotene ^b	Petroleum ether	418	396	376		
OH-zeta-carotene ^b	Petroleum ether	417	396	376		
di-OH-zeta-carotene ^b	Petroleum ether	420	397	378		
θ -carotene ^c	Not specified	421	397	375.5		
Fraction I	Diethyl ether	418	397*	378*	360	338
Fraction II-a	Diethyl ether	418	396*	375*		
Fraction II-b	Methanol	414	397*	378		
Fraction III	Methanol		397			
Calculated with Kuhn's formula	None	408				

* Indicates λ_{max} .

^a Nash, 1945.

^b Jensen, 1958.

^c Haxo, 1955.

When the ether solution of Fraction II-a was partitioned against alkali, the pigment was distributed between the two phases. Acidification moved all pigment into the epiphase. Hence Fraction II-a may contain at least two components, only one of which has an acidic group.

When partitioned against ether and alkali, all pigments in Fractions II-b and III were hypophasic. Addition of methanol moved very little pigment into the epiphase. Acidification moved the pigments into the epiphase. Therefore, the pigments of Fractions II-b and III possess acidic functions.

Test for an acidic function in conjugation with the chromophore

The positions of the maxima of Fractions I, II-a, and II-b in the visible region were not altered by pH. The intensity of absorption, however, increased in basic solutions and decreased in acidic solutions.

The maximum of Fraction III occurred at 392 $m\mu$ in alkaline methanol but was

at 397 $m\mu$ in acidic methanol. The absorption increased in alkaline methanol and decreased in acidic methanol. The spectral shift of 5 $m\mu$ is consistent with the data for neurosporaxanthin (Zalokar, 1957) and indicates that the acidic function is conjugated with the chromophore.

Qualitative tests for polyene structure

The tests in Fractions I and II-a were all weakly positive; treatment with H_2SO_4 produced traces of blue which rapidly gave way to relatively stable brown colors. The Carr-Price reaction yielded deep bluish-orange colors. The color of these fractions was not entirely abolished by dithionite, although absorption in the visible disappeared. On the other hand, the brilliant blue colors obtained by treating Fractions II-b and III with H_2SO_4 (Haxo, 1949; Karrer and Jucker, 1950), and the complete decolorization of these two fractions by dithionite showed polyene structures.

TABLE II

The effect of various concentrations of DPA upon mature sorocarp formation and pigment synthesis in D. discoideum

Concentration of DPA in media	Relative number of sorocarps	Relative pigment concentration
$5 \times 10^{-6} M$	None	—
$4 \times 10^{-6} M$	Very few	Colorless
$3 \times 10^{-6} M$	Not abundant	Colorless to pale yellow
$2 \times 10^{-6} M$	Abundant	Colorless to pale yellow
$1 \times 10^{-6} M$	Abundant	Pale yellow
$5 \times 10^{-6} M$	Abundant	Only slightly less yellow than controls
Ethanol control	Abundant	Bright lemon yellow
Control	Abundant	Bright lemon yellow

The results suggest that Fractions I and II-a contained carotenoid pigments, with, however, colorless impurities and non-carotenoid pigments of unknown nature. The data point to many similarities between these non-carotenoid pigments and the lipofuschins described by Fox (1953). Unquestionably the pigments in Fractions II-b and II are carotenoids.

The effect of diphenylamine on pigment synthesis

The effects of several concentrations of DPA upon pigment synthesis are given in Table II. At $2 \times 10^{-6} M$ to $3 \times 10^{-6} M$ it significantly inhibited pigment synthesis without interfering with growth. This concentration range was very critical. Growth was severely limited at $5 \times 10^{-6} M$ DPA, but in the presence of $5 \times 10^{-6} M$ DPA the mature sorocarps were practically indistinguishable from the controls.

DISCUSSION

Whittingham and Raper (1956) have suggested that pigmentation in *D. discoideum* sori depends upon environmental factors, such as the substratum or the bacterial associate upon which the slime mold feeds. Other studies have estab-

lished that sorocarp color may be influenced by the incorporation of vital dyes (Bonner, 1952) or pigmented foodstuffs (Raper, 1937). In these instances pigmentation occurs as a result of the accumulation and retention of soluble or particulate exogenous pigment.

This investigation indicates that sorocarp pigmentation arises by *de novo* synthesis. Absorption spectra of ethanol extracts of *D. discoideum* showed in the visible region a characteristic peak which was absent from ethanol extracts of both *E. coli* and nutrient agar (Fig. 2). Further, a sharp separation exists between the feeding period and the morphogenetic phase of the life cycle (Bonner, 1947, 1959). Although food intake ceases at the beginning of aggregation (Bonner, 1959), the colorless pseudoplasmodia could be transferred to a non-nutrient agar surface where pigmented fruiting bodies subsequently occurred. Further evidence that the yellow pigment is not merely accumulated was obtained from the studies relating pigment concentration to development (Fig. 1). At the time pigment appears in significant quantities, the spore cells are supported in the air by a stalk, and thus are removed from immediate contact with any exogenous supply of pigment.

These pigments are mainly carotenoids. At a suitable concentration, DPA, a well known inhibitor of carotenoid synthesis, either decreased or completely inhibited pigmentation without affecting growth. In addition, the massive bands in the visible region of the absorption spectra are characteristic of carotenoid pigments. This band, believed to arise from the oscillation of pi electrons from one end of the conjugated polyene structure composing the chromophore to the other (Dale, 1954), often exhibits fine structure usually manifested by three maxima or two maxima and a shoulder. The peak with the highest intensity is referred to as the λ_{\max} , while the whole band, including its fine structure, is referred to as the fundamental band or λ_1 (Dale, 1954; Zechmeister, 1960). For a given solvent, the position of this band and its degree of fine structure depend upon the length of the chromophore (Zechmeister, 1960). One of the most important determinants of the length of the chromophore and hence the position of λ_1 is the number of conjugated double bonds. This relationship has been worked out both theoretically and empirically so that by the position of λ_1 , one can estimate rather closely the number of conjugated double bonds in the chromophore. From curves relating the number of conjugated double bonds and maxima at λ_1 (Dale, 1954; Nash, 1948), it was estimated that pigments in Fractions I, II-a, II-b, and III possessed seven conjugated double bonds. Then, Kuhn's empirical formula was used to calculate the wave-lengths near which a system with seven double bonds should display maxima (Table I). Kuhn's formula (Dale, 1954) is:

$$\lambda_s = \frac{157 \text{ m}\mu}{\sqrt{1 - 0.922 \cos\left(\frac{\pi(s)}{n + 1}\right)}}$$

where n = the number of conjugated double bonds and s = the band order. It can be seen that close agreement exists between observed and calculated values at λ_1 , despite the dependence of the position of this band upon both the solvent and the presence or absence of an oxygen atom conjugated with the chromophore. The observed maxima at λ_1 are compared with literature values of known carotenoids in Table I.

The evidence that these pigments are acidic is of interest, for relatively few acidic carotenoids have been described. In the native state these pigments exhibited limited solubility in water. It is unlikely that combination with protein contributed in any substantial way to this water-solubility, for a dramatic spectral shift (Fox, 1948) was never observed when the pigments were subjected to procedures which would hydrolyze a protein moiety and liberate pigment. On the other hand, esterification with a sugar residue may be an important factor in conferring this limited water-solubility. Until the pigments were hydrolyzed, no amount of acid forced the pigments from the ethanol extract into the ether phase.

The partition behavior of these pigments was not studied extensively. However, the insolubility of Fractions II-b and III in such non-polar solvents as hexane, benzene, and carbon disulfide suggested the presence of polar groups. The possibility that these pigments were xanthophylls is of interest with respect to the observed DPA inhibition. Haxo (1955) reported that DPA inhibited formation of xanthophylls in *Mycobacterium phlei*, and Turian and Haxo (1952) found this inhibition to be most marked at the terminal synthetic steps, *i.e.*, at the conversion of neutral hydrocarbons into acidic compounds.

The evidence that these pigments are carotenoids and that they contain seven conjugated double bonds leads to the conclusion that they probably belong to the zeta-carotene series.

Functional significance of these pigments should be explored. Most attempts to establish a function of carotenoids in fungi have pointed to their mediating photokinetic responses (Goodwin, 1954). Migrating pseudoplasmodia in *D. discoideum* exhibit a strong positive photoactive response (Bonner, 1952); and Francis (1964) has recently shown this action spectrum to have a major peak near 425 $m\mu$ and a minor peak near 550 $m\mu$. He found that the absorption spectrum of "slime" from spore heads also peaked about 425 $m\mu$. On the basis of these two findings, he has suggested that the slime sheath may contain the receptor system for phototaxis. The absorption spectrum of sori "slime" which Francis has reported is similar to the spectra of pigments studied in this investigation, and it seems plausible that these components are identical. Although Francis has demonstrated a phototactic response in the migrating pseudoplasmodia, he did not study the absorption spectra of this stage. In the present study no absorption peak near 395 $m\mu$, and consequently no yellow pigment, was found in migrating pseudoplasmodia. In order to demonstrate an association between phototaxis and a pigment, one should be able to correlate the absorption spectrum maximum with the same developmental stage as that in which the phototactic response occurs. Evidence obtained by other investigators (Goodwin, 1952, 1954) suggests that in some instances as little as 1-2% of the usual carotene content may be sufficient for phototactic action; or alternatively, that the more saturated polyenes mediate a photokinetic response. Another possibility is that the pigment and phototactic response have nothing to do with each other.

There is a great deal of circumstantial evidence in the literature pointing to, but never specifically defining, a reproductive function for carotenoids. In *D. discoideum*, carotenoids accumulate in the spores, *i.e.*, in the structures directly concerned with reproduction. The pigmentation reaches a peak after fruiting and sharply decreases in the vegetative phase. These findings are similar to those of

other investigators (Emerson and Fox, 1940; Fox, 1948; Goodwin, 1950; Murneek, 1934) which indicate that the highest concentrations of carotenoids in plants and animals are found in tissues and secretions associated with reproduction, and suggest that carotenoids for some reason may be associated with reproduction in *D. discoideum* although no conclusions about their function can be drawn from the present evidence.

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SUMMARY

1. The lemon-yellow pigmentation in the mature sori of the cellular slime mold, *Dictyostelium discoideum*, was shown to arise by *de novo* synthesis and not by accumulation from an exogenous source. Pigment synthesis reached a peak after fruiting and then sharply declined in the vegetative phase.

2. The major pigments appeared to be related to the zeta-carotenes. Inhibition of pigment synthesis by diphenylamine, which specifically inhibits carotenogenesis, indicated the pigments were carotenoids. Chemical and spectral analyses of the pigments indicated polyene structures with seven conjugated double bonds.

3. Most of the pigments contained acidic functions. The acidic function of one pigment appeared to be conjugated with the chromophore.

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